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Isolation of a primate embryonic stem cell line

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ABSTRACT **Embryonic stem cells have the ability to remain undifferentiated and proliferate indefinitely *in vitro* while maintaining the potential to differentiate into derivatives of all three embryonic germ layers. Here we report the derivation of a cloned cell line (R278.5) from a rhesus monkey blastocyst that remains undifferentiated in continuous passage for >1 year, maintains a normal XY karyotype, and expresses the cell surface markers (alkaline phosphatase, stage-specific embryonic antigen 3, stage-specific embryonic antigen 4, TRA-1-60, and TRA-1-81) that are characteristic of human embryonal carcinoma cells. R278.5 cells remain undifferentiated when grown on mouse embryonic fibroblast feeder layers but differentiate or die in the absence of fibroblasts, despite the presence of recombinant human leukemia inhibitory factor. R278.5 cells allowed to differentiate *in vitro* secrete bioactive chorionic gonadotropin into the medium, express chorionic gonadotropin α- and β-subunit mRNAs, and express α-fetoprotein mRNA, indicating trophoblast and endoderm differentiation. When injected into severe combined immunodeficient mice, R278.5 cells consistently differentiate into derivatives of all three embryonic germ layers. These results define R278.5 cells as an embryonic stem cell line, to our knowledge, the first to be derived from any primate species.**

Embryonic stem (ES) cells, derived from preimplantation embryos (1, 2), and embryonic germ (EG) cells, derived from fetal germ cells (3, 4), are undifferentiated, immortal cells capable of differentiating into derivatives of all three embryonic germ layers. Well-characterized ES and EG cells have been derived only from rodents (1, 2, 5, 6). Pluripotent cell lines have been derived from preimplantation embryos of several non-rodent species (7–10), but the developmental potentials of these cell lines remain poorly characterized. Mouse ES cells remain undifferentiated through serial passages when cultured in the presence of leukemia inhibitory factor (LIF) and differentiate in the absence of LIF (11). Mouse ES cells injected into syngeneic mice form teratocarcinomas that exhibit disorganized differentiation, with representatives of all three embryonic germ layers. Mouse ES cells combined with normal preimplantation embryos as chimeras and returned to the uterus participate in normal development (12). Because mouse ES cells can contribute to functional germ cells in chimeras, specific genetic changes can be introduced into the mouse germ line through the use of ES cell chimeras (13).

The mechanisms controlling differentiation of specific lineages can be studied with mouse ES cells grown *in vitro*; however, significant differences between early human and mouse development suggest that human development will be more accurately represented by primate ES cells. For example, human and mouse embryos differ in the timing of embryonic genome expression (14), in the structure and function of the fetal membranes and placenta (15), and in formation of an embryonic disc instead of an egg cylinder. Human embryonal carcinoma (EC) cells, which are pluripotent, immortal stem cells from teratocarcinomas, provide an important *in vitro* model for understanding human differentiation (16). Some EC cell lines can be induced to differentiate in culture (17), which results in the loss of specific cell surface markers [stage-specific embryonic antigen 3 (SSEA-3), SSEA-4, TRA-1-60, and TRA-1-81] and the appearance of new markers (16). When pluripotent human EC cells are injected into immunocompromised mice, they form teratocarcinomas, some with derivatives of all three embryonic germ layers. However, there are limitations to the use of human EC cells in the study of development. (*i*) The range of differentiation obtained from human EC cell lines is more limited than that obtained from mouse ES cells and varies widely between cell lines (18). (*ii*) All pluripotent human EC cell lines derived to date are aneuploid (19), suggesting EC cells may not provide a completely accurate representation of normal differentiation. (*iii*) Ethical considerations severely restrict the study of human embryos, often making it impossible to verify that *in vitro* results have significance in the intact embryo. None of these limitations would be present with nonhuman primate ES cell lines.

Here we report the isolation of an ES cell line (R278.5) from a rhesus monkey blastocyst. This cloned cell line remains undifferentiated and continues to proliferate for >1 year in culture, maintains a normal XY karyotype, and maintains the potential to differentiate into trophoblast and to derivatives of embryonic endoderm, mesoderm, and ectoderm. The morphology, cell surface markers, and growth factor requirements of these cells differ significantly from mouse ES cells but closely resemble human EC cells.

MATERIALS AND METHODS

Cell Line Isolation. Six days after ovulation, an azonal blastocyst was recovered by a nonsurgical uterine flush technique from a 15-year-old rhesus monkey (20). The trophectoderm was removed by immunosurgery (21) using a rabbit anti-rhesus spleen cell antiserum followed by exposure to guinea pig complement. The intact inner cell mass (ICM) was separated from lysed trophectoderm cells and plated on mouse embryonic fibroblasts [previously exposed to 3000 rads (1 rad = 0.01 Gy) γ-radiation] in medium consisting of 80% Dulbecco's modified Eagle medium (4500 mg of glucose per liter, with L-glutamine, without sodium pyruvate; GIBCO) with 20% fetal bovine serum (HyClone), 0.1 mM 2-mercaptoethanol (Sigma), 1% nonessential amino acid stock (GIBCO) (22), and 1000 units of cloned human LIF per ml (GIBCO). After 16 days of culture, a central mass of cells was removed from

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Abbreviations: CG, chorionic gonadotropin; ES, embryonic stem; EC, embryonal carcinoma; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ICM, inner cell mass; LIF, leukemia inhibitory factor; RT-PCR, reverse transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SSEA, stage-specific embryonic antigen.

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epithelial outgrowths, exposed for 3 min to 0.05% trypsin-EDTA (GIBCO), gently dissociated by pipetting through a micropipette, and replated on mouse embryonic fibroblasts. After 3 weeks of growth, colonies with a morphology resembling human EC cells were selected and expanded. At five passages, individual cells were selected by micropipette and plated in individual wells of a 96-well plate (Falcon) with mouse embryonic fibroblast feeder layers. One clone with a normal karyotype (R278.5) was expanded for further analysis.

Cell Surface Markers. R278.5 cells grown on a layer of mouse embryonic fibroblasts were used to examine the expression of cell surface markers. Alkaline phosphatase was detected histochemically following fixation of cells with 100% ethanol using "Vector red" (Vector Laboratories) as a substrate, as described by the manufacturer. The SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 antigens were detected by immunocytochemistry with specific primary monoclonal antibodies (gifts of Peter Andrews, University of Sheffield, U.K.) (16, 23–25) and localized with a biotinylated secondary antibody and then an avidin/biotinylated horseradish peroxidase complex (Vectastain ABC system, Vector Laboratories).

***In Vitro* Differentiation.** R278.5 cells were plated at low density (≈5000 cells/cm^2 of surface area) in the absence of fibroblasts on gelatin-treated four-well tissue culture plates (Nunc) in the same medium as that used for initial cell line isolation, but with 0–10^4 units of added human LIF per ml (GIBCO). The resulting differentiated cells were photographed 8 days after plating.

A mouse Leydig cell bioassay (26) was used to measure luteinizing hormone/chorionic gonadotropin (CG) activity in medium conditioned for 2 days either by undifferentiated R278.5 cells (at 80% confluence on fibroblast feeder layers) or by spontaneously differentiated R278.5 cells (cultured for 2 weeks after achieving confluence on fibroblast feeders). The relative levels of the mRNAs for α-fetoprotein and the α- and β-subunits of CG relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (27) using RNA from the same undifferentiated and differentiated cells. The PCR primers for human G3PDH (Clontech) do not amplify mouse G3PDH mRNA. Primers for human α-fetoprotein mRNA flank the seventh intron (5′ primer, 5′-GCTGGATTGTCTGCAGGATGGGGAA; 3′ primer, 5′-TCCCCTGAAGAAAATTGGTTAAAAT) and amplify a cDNA of 216 bp. Primers for the β-subunit of human CG flank the second intron (5′ primer, 5′-*ggatc*CACCGTCAACACCACCATCTGTGC; 3′ primer, 5′-*ggatc*CACAGGTCAAAGCGGTGGTCCTTGGG) (nucleotides added to the CGβ sequence to facilitate subcloning are shown in italics) and amplify a cDNA of 262 bp. The primers for the CGα subunit were based on sequences of the first and fourth exon of the rhesus gene (28) (5′ primer, 5′-*gggaattc*GCAGTTACTGAGAACTCACAAG; 3′ primer, 5′-*gggaattc*GAAGCATGTCAAAGTGGTATGG) and amplify a cDNA of 556 bp. The identity of all cDNAs was verified by sequencing (not shown).

For RT-PCR, 1–5 μl of total R278.5 RNA was reverse transcribed, and 1–20 μl of reverse transcription reaction was subjected to the PCR in the presence of 2.5 μCi of deoxycytidine 5′-[α-^{32}P]triphosphate (1 Ci = 37 GBq; DuPont). The number of amplification rounds that produced linear increases in target cDNAs and the relation between input RNA and amount of PCR product were empirically determined. Following agarose gel electrophoresis, DNA bands of interest were cut out and radioactivity was determined by liquid scintillation spectroscopy. The ratio of cpm in a specific PCR product relative to cpm of G3PDH PCR product was used to estimate the relative levels of mRNAs among differentiated and undifferentiated cells.

Tumor Formation in Severe Combined Immunodeficient (SCID) Mice. In the passage immediately prior to SCID mouse injection (7 months after initial derivation of R278), karyotypes of R278.5 were confirmed as euploid. Approximately 5 × 10^5 R278.5 cells were injected either into the rear leg muscles (seven mice) or into the testis (two mice) of 8- to 12-week-old male SCID mice. The resulting tumors were fixed in 4% paraformaldehyde and examined histologically after paraffin embedding at 8–15 weeks of development.

RESULTS

The morphology and cell surface markers of R278.5 cells (Fig. 1*A*) more closely resembled human EC cells than mouse ES cells. R278.5 cells had a high nucleus/cytoplasm ratio and prominent nucleoli, but rather than forming compact, piled-up colonies with indistinct cell borders similar to mouse ES cells, R278.5 cells formed flatter colonies with individual, distinct cells. R278.5 cells expressed alkaline phosphatase activity and the cell surface antigens SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 2), cell surface markers characteristic of human EC cell lines (16). Although cloned human LIF was present in the medium at cell line derivation and for initial passages, R278.5 cells grown on mouse embryonic fibroblasts without exogenous LIF remained undifferentiated and continued to proliferate. R278.5 cells plated on gelatin-treated tissue culture plates without fibroblasts differentiated to multiple cell types or failed to attach and died, regardless of the presence or absence of exogenously added human LIF (Fig. 1*B*).

The mRNA for α-fetoprotein, a marker for endoderm, increased substantially with *in vitro* differentiation (Fig. 3). α-Fetoprotein is expressed by extra-embryonic (yolk sac) and embryonic (fetal liver and intestines) endoderm. Epithelial cells resembling extraembryonic endoderm were present in cells differentiated *in vitro* from R278.5 cells (Fig. 1*B*).

Luteinizing hormone activity, an indication of CG secretion and trophoblast differentiation, was present in culture medium collected from differentiated cells [3.89 milli-international units (mIU)/ml] but not in medium collected from undiffer-

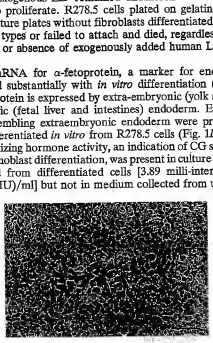

FIG. 1. Colony morphology and *in vitro* differentiation of cell line R278.5. (*A*) Undifferentiated R278.5 cells. Note the distinct cell borders, high nucleus to cytoplasm ratio, and prominent nucleoli. (Bar = 100 μm.) (*B*) Differentiated cells 8 days after plating R278.5 cells on gelatin-treated tissue culture plastic, with 10^3 units of added human LIF per ml. (Bar = 100 μm.)

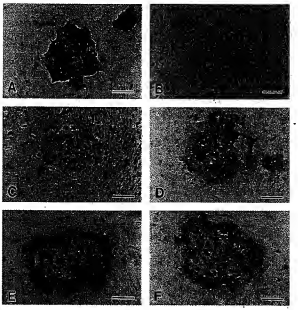

FIG. 2. Expression of cell surface markers by undifferentiated R278.5 cells. (*A*) Alkaline phosphatase. (*B*) SSEA-1. (*C*) SSEA-3. (*D*) SSEA-4. (*E*) TRA-1-60. (*F*) TRA-1-81. (Bars = 100 μm.) SSEA-3 staining of R278.5 cells was consistently weaker than the other positive antigens, and cell staining intensity varied within and between colonies.

entiated cells (<0.03 mIU/ml). The mRNAs for the CG subunits were readily detectable in the differentiated cells, although the relative level of the CGβ subunit mRNA was considerably lower than that for the CGα subunit (Fig. 4). The relative level of the CGα mRNA was quite low in undifferentiated cells, but the relative level was increased 23.9-fold after differentiation. The levels of the CGβ mRNA, on the other hand, increased only about 2-fold after differentiation for 2 weeks. Minor subpopulations of R278.5 cells differentiated even in the presence of fibroblasts, and the low level of α-fetoprotein, CGα, and CGβ mRNA present prior to the removal from fibroblasts could have been from these cells.

All SCID mice injected with R278.5 cells in either intramuscular or intratesticular sites formed tumors, and tumors in both sites demonstrated a similar range of differentiation. The

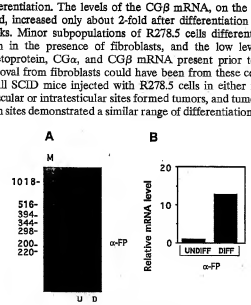

FIG. 3. Expression of α-fetoprotein mRNA. (*A*) PCR amplification of α-fetoprotein (αFP) cDNA from reverse-transcribed total RNA from undifferentiated (U) and differentiated (D) R278.5 cells. The DNA size markers (*M*) are indicated in bp. (*B*) The α-fetoprotein mRNA levels are expressed relative to the levels of the mRNA for G3PDH in each sample (not shown) as described in the text. Similar results were obtained in a second independent differentiation experiment.

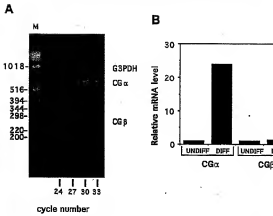

FIG. 4. Expression of CG subunit mRNA. (*A*) PCR amplification of cDNAs for G3PDH, CGα, and CGβ subunits from reverse-transcribed total RNA from differentiated R278.5 cells. The DNA size markers (M) are indicated in bp. (*B*) Relative levels of CGα and CGβ mRNAs in undifferentiated and differentiated R278.5 cells. Total RNA from cultured cells was analyzed for CG mRNA levels by RT-PCR and expressed relative to the levels of G3PDH mRNA. Similar results were obtained in a second independent differentiation experiment.

oldest tumors examined (15 weeks) had the most advanced differentiation, and all had abundant, unambiguous derivatives of all three embryonic germ layers, including ciliated columnar epithelium and nonciliated columnar epithelium (probable respiratory and gut epithelium; endoderm); bone, cartilage, smooth muscle, striated muscle (mesoderm); ganglia, other neural tissue, and stratified squamous epithelium (ectoderm), and other unidentified cell types (Fig. 5). Neural tissue included stratified cellular structures with remarkable resemblance to developing neural tube (Fig. 5*D*). Gut-like structures were often encircled by multiple layers of smooth muscle and were sometimes lined by villi with columnar epithelium interspersed with scattered mucus-secreting goblet cells (Fig. 5 *A* and *F*). Stratified squamous epithelium often contained well-differentiated hair follicles with hair shafts (Fig. 5*C*).

DISCUSSION

To our knowledge, there have been no previous reports of the isolation of a primate ES cell line. The characteristics that define R278.5 cells as ES cells include indefinite (>1 year) undifferentiated proliferation *in vitro*, maintenance of a normal karyotype, and potential to differentiate to derivatives of trophectoderm and all three embryonic germ layers. The development of complex structures in tumors in SCID mice with remarkable resemblance to normal hair follicles, neural tube, and gut demonstrates the ability of R278.5 cells to participate in complex developmental processes requiring coordinated interactions between multiple cell types. In the mouse embryo, the last cells capable of contributing to derivatives of trophectoderm and ICM are early ICM cells of the expanding blastocyst (29). The timing of commitment to ICM or trophectoderm has not been established for any primate species, but the potential of R278.5 cells to contribute to derivatives of both suggests that they most closely resemble early totipotent embryonic cells. The very limited ability of mouse ES cells to contribute to trophoblast in chimeras (30) suggests that the R278.5 cells represent an earlier developmental stage than mouse ES cells or that the ability of ICM cells to form trophectoderm persists longer in primates. Human EC cells share the ability of R278.5 cells to differentiate to trophoblast *in vitro* (16) and this potential may be a general distinguishing property of primate ES cell lines.

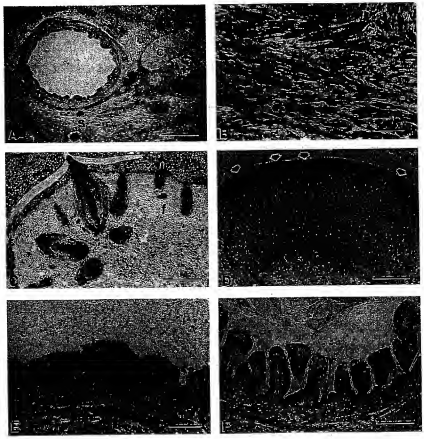

FIG. 5. Tumors formed by R278.5 cells injected into SCID mice and examined at 15 weeks. (*A*) Low-power field demonstrating disorganized differentiation of multiple cell types. A gut-like structure is encircled by smooth muscle (s), and elsewhere foci of cartilage (c) are present. (Bar = 400 μm.) (*B*) Striated muscle. (Bar = 40 μm.). (*C*) Stratified squamous epithelium with several hair follicles. The labeled hair follicle (f) has a visible hair shaft. (Bar = 200 μm.). (*D*) Stratified layers of neural cells in the pattern of a developing neural tube. An upper "ventricular" layer, containing numerous mitotic figures (arrows), overlies a lower "mantle" layer. (Bar = 100 μm.) (*E*) Ciliated columnar epithelium. (Bar = 40 μm.) (*F*) Villi covered with columnar epithelium with interspersed mucus-secreting goblet cells. (Bar = 200 μm.)

The only cells known to express the combination of markers alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 other than R278.5 cells are human EC cells (16, 25, 31). This expression pattern contrasts with undifferentiated mouse ES and EC cells, which instead express SSEA-1 and do not express SSEA-3, SSEA-4, TRA-1-60, or TRA-1-81 (23, 24). Differentiation of human EC cells such as NTERA 2 cl.D1 (17) results in the loss of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 expression and an increased SSEA-1 expression (16). These antigens have yet to be studied in early human or nonhuman primate embryos, and their functions are unknown, but their shared expression by R278.5 cells and human EC cells suggests a close embryological similarity.

In the absence of fibroblast feeder layers, soluble LIF fails to prevent the differentiation of R278.5 cells or of feeder-dependent human EC cells (19). The factors that fibroblasts produce that prevent the differentiation of R278.5 cells or feeder-dependent human EC cells are unknown. Other factors that fail to support the growth of feeder-dependent human EC cells in the absence of feeder layers include oncostatin M and ciliary neurotrophic factor (19), both of which can substitute for LIF in preventing the differentiation of mouse ES cells (32, 33). A trypsin-sensitive factor from a human yolk sac carcinoma cell line (GCT 44) supports the growth of feeder-dependent human EC cells in the absence of fibroblasts, but the factor has not yet been purified (19).

Although exogenous LIF was added during the initial derivation of R278.5 cells, the cell line is now routinely passaged without added LIF. We have also recently derived two additional cell lines (R366 and R367) from four additional rhesus blastocysts, using the same techniques as described for R278.5 cells, but without added LIF (data not shown). R366 and R367 cells have normal karyotypes and continue to proliferate *in vitro* for at least 3 months. R366 and R367 cell lines have not yet been tested for tumor formation in SCID mice, but they are indistinguishable from R278.5 cells in undifferentiated morphology, growth characteristics, and *in vitro* differentiation in the absence of feeder layers.

The differentiation of R278.5 cells to trophoblast was demonstrated by the expression of CGα and CGβ subunit mRNAs and the secretion of bioactive CG into the culture medium by differentiated ES cells. We were surprised to note that while the relative levels of the CGα subunit were increased >20 times in differentiated cells, the relative levels of the CGβ subunit only changed about 2-fold. The fact that CG secretion increased substantially with differentiation may mean that under our *in vitro* culture conditions, expression of the CGα subunit is limiting for CG secretion. CGβ subunit mRNA is detectable in human preimplantation embryos as early as the eight-cell stage, which is before trophectoderm differentiation (34), consistent with a low level of CGβ mRNA expression in undifferentiated R278.5 cells. Although there may be some coordinate mechanisms regulating CGα and CGβ gene transcription in the placenta (35), it is clear that there is differential regulation of these genes *in vitro* and *in vivo* (36). Since the expression of the CGβ subunit is also divergent among villous

and extravillous trophoblasts (37), further studies are needed to determine the phenotype of the trophoblasts derived from R278.5 cells.

Primate ES cells will be particularly useful for *in vitro* developmental studies of lineages that differ substantially between humans and mice. However, the most accurate *in vitro* model of the differentiation of human tissues would be provided by human ES cells. In one published report, ICM-derived cells from spare *in vitro* fertilized human embryos were cultured with LIF in the absence of feeder layers, and, although alkaline phosphatase positive cells proliferated, they failed to survive beyond two passages (38). These results suggest that soluble LIF alone will not prevent the differentiation of human ES cells, just as it fails to prevent the differentiation of rhesus ES cells. The growth of rhesus monkey ES cells in culture conditions that support feeder-dependent human EC cells suggests that similar conditions may support human ES cells.

Human ES cells would offer exciting new possibilities for transplantation medicine. Because ES cells have the developmental potential to give rise to all adult cell types, any disease resulting from the failure of specific cell types would be potentially treatable through the transplantation of differentiated cells derived from ES cells. Because ES cells are immortal cell lines, they could be genetically manipulated prior to differentiation either to reduce immunogenicity or to give them new properties to combat specific diseases. Rhesus monkey ES cells and rhesus monkeys will be invaluable for testing the safety and efficacy of the transplantation of specific cell types for the treatment of specific diseases. Because of the range of diseases potentially treatable by this approach, elucidating the basic mechanisms controlling the differentiation of primate ES cells has dramatic clinical significance.

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Embryonic Stem Cell Lines Derived from Human Blastocysts

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Human blastocyst-derived, pluripotent cell lines are described that have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages. After undifferentiated proliferation in vitro for 4 to 5 months, these cells still maintained the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). These cell lines should be useful in human developmental biology, drug discovery, and transplantation medicine.

Embryonic stem (ES) cells are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation in vitro (*1*, *2*). In chimeras with intact embryos, mouse ES cells contribute to a wide range of adult tissues, including germ cells, providing a powerful approach for introducing specific genetic changes into the mouse germ line (*3*). The term "ES cell" was introduced to distinguish these embryo-derived pluripotent cells from teratocarcinoma-derived pluripotent embryonal carcinoma (EC) cells (*2*). Given the historical introduction of the term "ES cell" and the properties of mouse ES cells, we proposed that the essential characteristics of primate ES cells should include (i) derivation from the preimplantation or periimplantation embryo, (ii) prolonged undifferentiated proliferation, and (iii) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture (*4*). For ethical and practical reasons, in many primate species, including humans, the ability of ES cells to contribute to the germ line in chimeras is not a testable property. Nonhuman primate ES cell lines provide an accurate in vitro model for understanding the differentiation of human tissues (*4*, *5*). We now describe human cell lines that fulfill our proposed criteria to define primate ES cells.

Fresh or frozen cleavage stage human embryos, produced by in vitro fertilization (IVF) for clinical purposes, were donated by individuals after informed consent and after institutional review board approval. Embryos were cultured to the blastocyst stage, 14 inner cell masses were isolated, and five ES cell lines originating from five separate embryos were derived, essentially as described for nonhuman primate ES cells (*5*, *6*). The resulting cells had a high ratio of nucleus to cytoplasm, prominent nucleoli, and a colony morphology similar to that of rhesus monkey ES cells (Fig. 1). Three cell lines (H1, H13, and H14) had a normal XY karyotype, and two cell lines (H7 and H9) had a normal XX karyotype. Each of the cell lines was successfully cryopreserved and thawed. Four of the cell lines were cryopreserved after 5 to 6 months of continuous undifferentiated proliferation. The other cell line, H9, retained a normal karyotype after 6 months of culture and has now been passaged continuously for more than 8 months (32 passages). A period of replicative crisis was not observed for any of the cell lines.

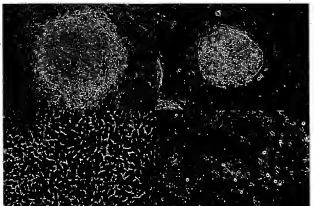

Fig. 1. Derivation of the H9 cell line. (**A**) Inner cell mass–derived cells attached to mouse embryonic fibroblast feeder layer after 8 days of culture, 24 hours before first dissociation. Scale bar, 100 μm. (**B**) H9 colony. Scale bar, 100 μm. (**C**) H9 cells. Scale bar, 50 μm. (**D**) Differentiated H9 cells, cultured for 5 days in the absence of mouse embryonic fibroblasts, but in the presence of human LIF (20 ng/ml; Sigma). Scale bar, 100 μm.

The human ES cell lines expressed high levels of telomerase activity (Fig. 2). Telomerase is a ribonucleoprotein that adds telomere repeats to chromosome ends and is involved in maintaining telomere length, which plays an important role in replicative life-span (*7*, *8*). Telomerase expression is highly correlated with immortality in human cell lines, and reintroduction of telomerase activity into some diploid human somatic cell lines extends replicative life-span (*9*). Diploid human somatic cells do not express telomerase, have shortened telomeres with age, and enter replicative senescence after a finite proliferative life-span in tissue culture (*10*–*13*). In contrast, telomerase is present at high levels in germ line and embryonic tissues (*14*). The high level of telomerase activity expressed by the human ES cell lines therefore suggests that their replicative life-span will exceed that of somatic cells.

The human ES cell lines expressed cell surface markers that characterize undifferentiated nonhuman primate ES and human EC cells, including stage-specific embryonic antigen (SSEA)–3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase (Fig. 3) (*4*, *5*, *15*, *16*). The globo-series glycolipid GL7, which carries the SSEA-4 epitope, is formed by the addition of sialic acid to the globo-series glycolipid Gb5, which carries the SSEA-3 epitope (*17*, *18*). Thus, GL7 reacts with antibodies to both SSEA-3 and SSEA-4 (*17*, *18*). Staining intensity for SSEA-4 on the human ES cell lines was consistently strong, but staining intensity for SSEA-3 was weak and varied both within and among colonies (Fig. 3, D and C). Because GL7 carries both the SSEA-4 and SSEA-3 epitopes and because staining for SSEA-4 was consistently strong, the relatively weak staining for

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SSEA-3 suggests a restricted access of the antibody to the SSEA-3 epitope. In common with human EC cells, the undifferentiated human ES cell lines did not stain for SSEA-1, but differentiated cells stained strongly for SSEA-1 (*15*) (Fig. 3). Mouse inner cell mass cells, ES cells, and EC cells express SSEA-1 but do not express SSEA-3 or SSEA-4 (*17*, *19*), suggesting basic species differences between early mouse and human development.

The human ES cell lines were derived by the selection and expansion of individual colonies of a uniform, undifferentiated morphology, but none of the ES cell lines was derived by the clonal expansion of a single cell. The uniform undifferentiated morphology that is shared by human ES and nonhuman primate ES cells and the consistent expression by the human ES cell lines of cell surface markers that uniquely characterize primate ES and human EC cells make it extremely unlikely that a mixed population of precursor cells was expanded. However, because the cell lines were not cloned from a single cell, we cannot rule out the possibility that there is some variation in developmental potential among the undifferentiated cells, in spite of their homogeneous appearance.

The human ES cell lines maintained the potential to form derivatives of all three embryonic germ layers. All five cell lines produced teratomas after injection into severe combined immunodeficient (SCID)–beige mice. Each injected mouse formed a teratoma, and all teratomas included gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm) (Fig. 4). In vitro, the ES cells differentiated when cultured in the absence of mouse embryonic fibroblast feeder layers, both in the presence and absence of human leukemia inhibitory factor (LIF) (Fig. 1). When grown to confluence and allowed to pile up in the culture dish, the ES cell lines differentiated spontaneously even in the presence of fibroblasts. After H9 cells were allowed to differentiate for 2 weeks, both α-fetoprotein (350.9 ± 14.2 IU/ml) and human chorionic gonadotropin (hCG, 46.7 ± 5.6 mIU/ml) were detected in conditioned culture medium, indicating endoderm and trophoblast differentiation (*20*).

Human ES cells should offer insights into developmental events that cannot be studied directly in the intact human embryo but that have important consequences in clinical areas, including birth defects, infertility, and pregnancy loss. Particularly in the early postimplantation period, knowledge of normal human development is largely restricted to the description of a limited number of sectioned embryos and to analogies drawn from the experimental embryology of other species (*21*). Although the mouse is the mainstay of experimental mammalian embryology, early structures including the placenta, extraembryonic membranes, and the egg cylinder all differ substantially from the corresponding structure of the human embryo. Human ES cells will be particularly valuable for the study of the development and function of tissues that differ between mice and humans. Screens based on the in vitro differentiation of human ES cells to specific lineages could identify gene targets for new drugs, genes that could be used for tissue regeneration therapies, and teratogenic or toxic compounds.

Elucidating the mechanisms that control differentiation will facilitate the efficient, directed differentiation of ES cells to specific cell types. The standardized production of large, purified populations of euploid human cells such as cardiomyocytes and neurons will provide a potentially limitless source of cells for drug discovery and

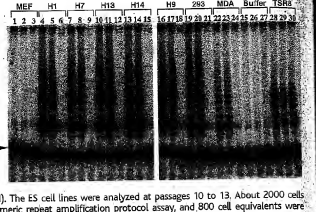

Fig. 2. Telomerase expression by human ES cell lines. MEF, irradiated mouse embryonic fibroblasts used as a feeder layer for the cells in lanes 4 to 18; 293, adenovirus-transformed kidney epithelial cell line 293; MDA, breast cancer cell line MDA; TSR8, quantitation control template. Telomerase activity was measured with the TRAPEZE Telomerase Detection Kit (Oncor, Gaithersburg, Maryland). The ES cell lines were analyzed at passages 10 to 13. About 2000 cells were assayed for each telomeric repeat amplification protocol assay, and 800 cell equivalents were loaded in each well of a 12.5% nondenaturing polyacrylamide gel. Reactions were done in triplicate with the third sample of each triplet heat inactivated for 10 to 15 min at 85°C before reaction to test for telomerase heat sensitivity (lanes 6, 9, 12, 15, 18, 21, 24, and 27). A 36–base pair internal control for amplification efficiency and quantitative analysis was run for each reaction as indicated by the arrowhead. Data were analyzed with the Storm 840 Scanner and ImageQuant package (Molecular Dynamics). Telomerase activity in the human ES cell lines ranged from 3.8 to 5.9 times that observed in the immortal human cell line MDA on a per cell basis.

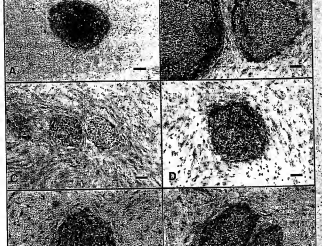

Fig. 3. Expression of cell surface markers by H9 cells. Scale bar, 100 μm. (**A**) Alkaline phosphatase. (**B**) SSEA-1. Undifferentiated cells failed to stain for SSEA-1 (large colony, left). Occasional colonies consisted of non-stained, central, undifferentiated cells surrounded by a margin of stained, differentiated, epithelial cells (small colony, right). (**C**) SSEA-3. Some small colonies stained uniformly for SSEA-3 (colony left of center), but most colonies contained a mixture of weakly stained cells and a majority of non-stained cells (colony right of center). (**D**) SSEA-4. (**E**) TRA-1-60. (**F**) TRA-1-81. Similar results were obtained for cell lines H1, H7, H13, and H14.

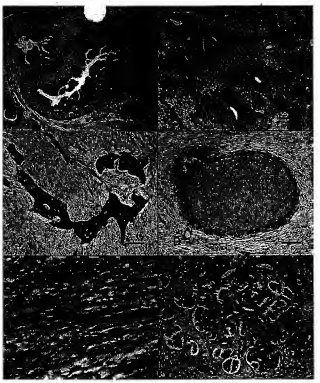

Fig. 4. Teratomas formed by the human ES cell lines in SCID-beige mice. Human ES cells after 4 to 5 months of culture (passages 14 to 16) from about 50% confluent six-well plates were injected into the rear leg muscles of 4-week-old male SCID-beige mice (two or more mice per cell line). Seven to eight weeks after injection, the resulting teratomas were examined histologically. (**A**) Cutlike structures. Cell line H9. Scale bar, 400 μm. (**B**) Rosettes of neural epithelium. Cell line H14. Scale bar, 200 μm. (**C**) Bone. Cell line H14. Scale bar, 100 μm. (**D**) Cartilage. Cell line H9. Scale bar, 100 μm. (**E**) Striated muscle. Cell line H13. Scale bar, 25 μm. (**F**) Tubules interspersed with structures resembling fetal glomeruli. Cell line H9. Scale bar, 100 μm.

transplantation therapies. Many diseases, such as Parkinson's disease and juvenile-onset diabetes mellitus, result from the death or dysfunction of just one or a few cell types. The replacement of those cells could offer lifelong treatment. Strategies to prevent immune rejection of the transplanted cells need to be developed but could include banking ES cells with defined major histocompatibility complex backgrounds or genetically manipulating ES cells to reduce or actively combat immune rejection. Because of the similarities to humans and human ES cells, rhesus monkeys and rhesus ES cells provide an accurate model for developing strategies to prevent immune rejection of transplanted cells and for demonstrating the safety and efficacy of ES cell–based therapies. Substantial advances in basic developmental biology are required to direct ES cells efficiently to lineages of human clinical importance. However, progress has already been made in the in vitro differentiation of mouse ES cells to neurons, hematopoietic cells, and cardiac muscle (*22–24*). Progress in basic developmental biology is now extremely rapid; human ES cells will link this progress even more closely to the prevention and treatment of human disease.

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6. Thirty-six fresh or frozen-thawed donated human embryos produced by IVF were cultured to the blastocyst stage in G1.2 and G2.2 medium (*25*). Fourteen of the 20 blastocysts that developed were selected for ES cell isolation, as described for rhesus monkey ES cells (*5*). The inner cell masses were isolated by immunosurgery (*26*), with a rabbit antiserum to BeWO cells, and plated on irradiated (35 grays gamma irradiation) mouse embryonic fibroblasts. Culture medium consisted of 80% Dulbecco's modified Eagle's medium (no pyruvate, high glucose formulation; Gibco-BRL) supplemented with 20% fetal bovine serum (Hyclone), 1 mM glutamine, 0.1 mM β-mercaptoethanol (Sigma), and 1% nonessential amino acid stock (Gibco-BRL). After 9 to 15 days, inner cell mass–derived outgrowths were dissociated into clumps either by exposure to Ca^{2+}/Mg^{2+}-free phosphate-buffered saline with 1 mM EDTA (cell line H1), by exposure to dispase (10 mg/ml; Sigma; cell line H7), or by mechanical dissociation with a micropipette (cell lines H9, H13, and H14) and replated on irradiated mouse embryonic fibroblasts in fresh medium. Individual colonies with a uniform undifferentiated morphology were individually selected by micropipette, mechanically dissociated into clumps, and replated. Once established and expanded, cultures were passaged by exposure to type IV collagenase (1 mg/ml; Gibco-BRL) or by selection of individual colonies by micropipette. Clump sizes of about 50 to 100 cells were optimal. Cell lines were initially karyotyped at passages 2 to 7.
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20. hCG and α-fetoprotein were measured by specific radioimmunoassay (double Ab hCG and AFP-TC kits; Diagnostic Products, Los Angeles, CA). hCG assays used the World Health Organization Third International Standard 75/537. H9 cells were allowed to grow to confluence (day 0) on plates of irradiated mouse embryonic fibroblasts. Medium was replaced daily. After 2 weeks of differentiation, medium in triplicate wells conditioned for 24 hours was assayed for hCG and α-fetoprotein. No hCG or α-fetoprotein was detected in unconditioned medium.
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